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(54) Vaccine comprising autologous epidermal growth factor and use thereof.

(57) The invention provides novel uses of EGF and vaccine compositions comprising EGF.
In particularly autologous EGF, or a fragment or a derivative thereof is used as an active immunisation against the proliferation of EGF-dependent tumours, or other EGF-dependent diseases.
Autologous EGF is preferably coupled to a carrier protein, such as Tetanus toxoid or Cholera toxin B chain.
The vaccine compositions according to the invention will usually comprise an adjuvant such as aluminium hydroxide.

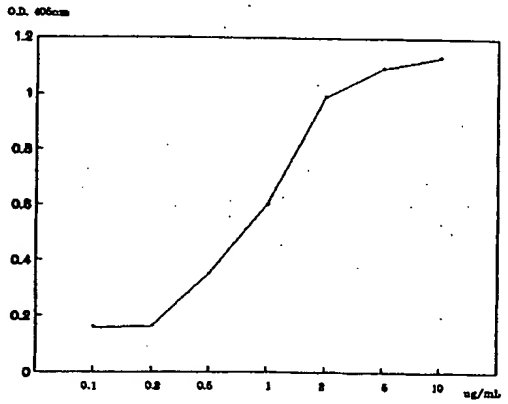

FIG.1

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since the art consistently reports that "self" molecules will not induce any immune reaction because the host has been educated to be tolerant to self.

The present invention provides a vaccine composition containing autologous EGF coupled to a carrier protein, which complex will inhibit the EGF dependent tumors' growth, through an autoimmune effect, without the collateral effects of the introduction of a heterologous protein in the human body.

This vaccine composition can be used in the treatment of EGF dependent tumors or any malignant disease associated to EGF.

It will be understood that in this specification EGF is to be read as including any fragment and/or derivative of EGF which has similar immunological properties and/or effects as the original molecule. Derivatives include, but are not limited to, conventional aminoacid substitutions, site-directed replacement of aminoacids for enhanced stability and/or activity, chemical modifications and the like.

DETAILED DESCRIPTION OF THE INVENTION:

I. OBTAINING AN IMMUNOGENIC PREPARATION:

Two preparations were obtained. The first is murine EGF (mu-EGF) coupled to a protein carrier and the second is human recombinant EGF (hu-re-EGF) (National Medicament Register Office from Cuba, HEBERMIN, No.1266), also coupled to a carrier protein.

The preparation containing hu-rec-EGF coupled to a carrier protein was obtained to be used only in non human primates and in humans. A proper adjuvant is applied.

The mu-EGF conjugated to a protein carrier is used in the studies performed in mice as a model to determine the immunogenicity and the antitumoral effect of a preparation containing an autologous EGF molecule.

Immunogenicity studies were performed in primates with the conjugate hu-EGF-carrier protein carrier since the hu-EGF is very similar to the primate EGF though it is recognized as a self molecule. These results allowed to demonstrate the immunogenic response an autologous molecule can elicit.

To obtain the preparations a solution of murine or hu-rec-EGF in PBS/$MgCl_2$ 10 mM, is mixed with a solution of the carrier protein in the same solvent, in a ratio of between 1 and 5 moles of EGF per mol of protein.

Afterwards glutaraldehyde 0.5%, is added to obtain a final concentration between 0.1% and 0.05%.

The mixture is incubated between 1 and 3 hours at room temperature and subsequently dialyzed in PBS/MgC12 10 mM with, at least, 3 changes of dialysis solution.

CONJUGATE CHARACTERIZATION:

The test of the conjugation efficiency and of the maintenance of the antigenicity is performed through an ELISA assay.

ELISA plates of activated PVC (NUNC) were coated with 50 µl of an antiserum against the carrier protein utilized, in a concentration between 1 and 10 µg/ml. In the case of cholera toxin chaim B (CTB) as carrier, the plates were coated with the ganglioside GMI.

Subsequently 3 washes with PBS/Tween were carried out and then the plates were blocked with a solution of BSA between 0.5 and 1% in PBS/Tween, incubated them during a period of 30 minutes to 1 hour at 37 °C. Dilutions between 0.1 and 0.001 mg/ml of the conjugates to be assayed were added to the plates, 50 µl/well, and incubated for 1 to 2 hours at 37 °C.

In the next step a mouse anti hu-rec-EGF antiserum was added in a dilution between 1:500 and 1:1000, 50 µl/well, and incubated for between 30 minutes and 1 hour at 37 °C.

As the last step, the plates were incubated with an anti-mouse-alkaline phosphatase antiserum, in a dilution between 1:500 and 1:1000, 50 µl/well, for 30 minutes to 1 hour at 37 °C.

Reaction color was developed with p-nitrophenylphosphate, at a concentration of 1 mg/ml in diethanolamine, 50 µl/well, incubated for 30 minutes at 37 °C. Optical density was measured at 405 nm in an ELISA plate reader.

The results demonstrated the activity of the molecule and the efficiency of the conjugation, because the conjugate maintains its recognition site for the molecule coating the plates, which specifically recognizes the carrier protein and, at the same time can be recognized by an anti EGF antiserum.

II.-CHARACTERIZATION OF EFFECTS PRODUCED BY THE PREPARATION CONTAINING mu-EGF. PRE-CLINICAL STUDIES.

IIa) ENDOGENOUS EGF IMMUNOGENICITY: INDUCTION OF AUTOIMMUNITY IN MICE.

In order to demonstrate the capacity of the immunogenic preparation containing mu-EGF obtained through the technique described in item I of inducing autoimmunity against the endogen EGF, a test was performed in Balb/C mice.

EXPERIMENTS

EXAMPLE 1.

STUDY OF THE PRESENCE OF AN EGF PRECURSOR MOLECULE IN THE CELL MEMBRANE OF EGF DEPENDENT TUMORS.

This study was performed through a Western Blotting technique. Samples of 5 ductal carcinoma of the breast in different stages, one head and neck tumor, four samples of fibrocystic dysplasia and five normal samples obtained as controls were studied.

Cell membranes were obtained from the samples through the procedure described elsewhere (Grimaux M.,Rev Neurol. 1988, 144: 101-103).

Electrophoresis was performed at 250 v, 10 mA, 3. OW, 15 °C and 150 Vh. Molecular weight standards were used in the range of 14 300 D (Lysozyme) to 340 000 D (alpha 2 macroglobulin).

Proteins separated during electrophoresis were transferred to a nitrocellulose membrane of 0.45 µm in a Phast system equipment in a buffer transfer solution. After the transference the membrane was blocked overnight with 10% skimmed milk with constant stirring.

After three washes in buffer solution a mouse monoclonal antibody recognizing human EGF was added and incubated during one hour.

After three washes a biotynilated anti mouse antibody was added and incubated during one hour. Peroxidase streptavidine conjugate was added and reaction developed with diaminobenzidine and hydrogen peroxide after one hour of incubation.

The results obtained demonstrated that the samples studied corresponding to normal tissues did not show any band in the zone of high molecular weight according to the standards. However, the samples corresponding to breast pathology (dysplasia and carcinomas) showed a diffuse banding in the high molecular weight zone. This is an experimental evidence of the presence of a high MW EGF precursor in the tumor membranes.

EXAMPLE 2

OBTAINING THE mu-EGF/CTB CONJUGATE:

One ml of mu-EGF in PBS/$MgCl_2$ 10 mM at a concentration of 1mg/ml, was mixed with 2 ml of a solution of CTB in the same solvent at a ratio of 1 mol of mu EGF per mol of CTB. Glutaraldehyde (3 ml, 0.5%) was added to obtain a final concentration of 0.05%.

Incubation was performed during 1 hour at room temperature and subsequently dialyzed in PBS/$MgCl_2$ 10 mM with, at least, 3 changes of the dialysis solution.

EXAMPLE 3

mu-EGF/CTB CONJUGATED CHARACTERIZATION:

ELISA assay for conjugate test: PVC activated ELISA plates (NUNC) were coated with 50 µl of the GM1 ganglioside (recognizing the CTB molecule) in a concentration of 4 µg/ml in methanol, which was left to dry off in the flow during 1 hour.

Subsequently 3 washes with PBS/Tween were carried out and then the plates were blocked with a solution of BSA 1% in PBS/Tween and incubated during 30 minutes at 37 °C.

Conjugated dilutions between 0.1 and 0.001 mg/ml were added to the plates at 50 µl/well and incubated during 1 hour at 37 °C.

Next a mouse anti-mu-EGF antiserum in a 1:1000 dilution, 50 µl/well was added and incubated for 1 hour at 37 °C.

Then, the plates were incubated with anti mouse antiserum alkaline phosphatase conjugate (dilution 1:1000), 50 µl/well for 1 hour at 37 °C. The color was developed with p-nitrophenylphosphate at a concentration of 1 mg/ml in diethanolamine, 50 µl/well, incubated for 30 minutes at 37 °C, optical density was measured at 405 nm.

The results demonstrated a direct relationship between the concentration of the conjugated and the absorbance values. This demonstrates the activity of the conjugated and the efficiency of the conjugation, since, the molecule maintains the recognition for the GM1 ganglioside (identifies CTB) and, at the same time, is recognized by an anti mu-EGF antiserum (Figure 1)

EXAMPLE 4.

IMMUNOGENICITY OF AUTOLOGOUS EGF: INDUCTION OF AUTO IMMUNITY IN MICE:

In order to demonstrate that the immunogenic preparation containing autologous EGF is capable of inducing autoimmunity, the experimentation was performed in Balb/c mice.

Groups of animals were inoculated with a dose of 50 µg of conjugated mu-EGF per animal subcutaneously weekly during 4 to 6 weeks.

The first week the immunogenic preparation was prepared in a proportion 1:1 with complete Freund's adjuvant, all the following doses were prepared with incomplete Freund's adjuvant.

The same procedure was performed in a control group, but only adjuvant was administered to the animals. One week after the last immunization,

coxon tests.

EXAMPLE 8

ASSOCIATION BETWEEN ANTIBODY TITER AGAINST mu EGF AND ^{125}I EGF BIODISTRIBUTION.

This experiment was performed to demonstrate that there is a different biodistribution of ^{125}I EGF in animals with antibody titer against mu- EGF in relation to animals that did not have antibody titer against mu-EGF.

An experiment with 4 groups of mice was performed for this purpose:

Group 1: 30 mice with antibody titer against mu-EGF.

Group 2: 30 mice without antibody titer against mu-EGF.

Group 3: 30 mice with antibody titer against mu-EGF grafted with EAT.

Group 4: 30 mice without antibody titer against mu-EGF grafted with EAT.

Samples from group 1 and 2 were taken from blood, lung, kidneys, liver and skin at the following times: 2, 5, 8, 11, 15, 20, 30, 60, 120 and 150 minutes and 3 animals were sacrificed at every corresponding time, counting the radioactivity in the organs extracted.

The results obtained have shown a difference in the accumulation of I-125 EGF in time mainly kidney and liver (Figure 6 a,b), indicated that the presence of antibodies against EGF do alter the biodistribution of this molecule.

The results obtained have also shown that there is a difference between the animals with or with without antibody titer (Figure 8,a,b,c,d) indicating that the animals with antibody titer can produce immunocomplexes with the circulating EGF showing a different depuration mechanism of the labelled EGF.

Samples from group 3 and 4 were taken from blood, lung, kidneys, liver, skin and from the ascitic fluid at the following times: 2, 5, 8, 11, 15, 20, 30, 60, 120 and 150 minutes and 3 animals were sacrificed at every corresponding time, and the radioactivity counted in the organs extracted.

It could be appreciated less accumulation of the labelled EGF in the ascitic fluid of animals with antibody titer than in the animals without antibody titer (Figure 7), indicating a more rapid depuration of the EGF present in the ascitic fluid in these animals and/or a limitation in EGF access to the ascites.

EXAMPLE 9:

IMMUNE RESPONSE CHARACTERIZATION: ISOTYPE OBTAINED AGAINST AUTOLOGOUS EGF.

In order to know whether the autoimmune response obtained upon the immunization of mice with the autologous EGF was a response producing antibodies of the isotype IgM or IgG, an ELISA assay was performed, in which the plates were coated with EGF to concentration of 10 µg/ml, 50 µg/well, and incubated for 1 hour at 37 °C.

Subsequently, dilutions between 1:10 and 1:1000 of the serums of animals immunized with mu EGF-CTB according to Example 5 were applied, 50 µg/well and were incubated for 1 hour at 37 °C.

Parallel design of plates were performed to measured IgG or IgM response with the corresponding antiserum (anti-IgG or anti-IgM respectively).

Color in the plates was developed with p-nitrophenyl phosphate, at a concentration of 1 mg/ml in diethanolamine, incubating during 30 minutes at 37 °C and values of optical density at 405 nm were read.

IgG response was obtained in all treated animals (Figure 8)

EXAMPLE 10

CHARACTERIZATION OF THE MEMORY OF THE IMMUNE RESPONSE AGAINST AUTOLOGOUS EGF.

Two groups of 10 mice were studied with a single immunization of 50 µg hu-re-EGF in complete Freund's adjuvant.

Group I.-The kinetics of antibody production against mu EGF was studied in this group of animals. Every 4 days blood samples were extracted. The antibody levels were determined by an ELISA technique.

Group II. - Was conformed with the animals with declining antibody titers from Group I and immunized again. Every 2 days blood samples were extracted. The antibody levels were determined by an ELISA technique.

Results have shown a memory response when the animals were immunized again with the preparation (Figure 9).

y axis: optical density at 405 nm

Figure 5 : Survival of animals immunized with mu EGF-CTB and subsequently inoculated with Ehrlich Ascites Tumour, in comparison with control animals (nonimmunized) and inoculated with same tumor.

Figure 6-a: Accumulation of 125-I EGF in liver of mice immunized with hu-rec-EGF in relation to nonimmunized controls.

Figure 6-b: Accumulation of 125-I EGF in kidneys of mice immunized with hu-rec-EGF in relation to nonimmunized mice.

Figure 7: Accumulation of mu-EGF I^{125}, in ascitic fluid of animals grafted with EAT, previously immunized with hu-rec-EGF.

Figure 8: ELISA assay for determination of IgG or IgM of the immune response in animals immunized with mu EGF-CTB.

Figure 9: Antibody response kinetic (IgG) against mu EGF, in animals immunized against hu-rec-EGF (Memory).

x axis: time (days).

y axis: Inverse logarithm of the antibody titer.(mean value).

Figure 10: ELISA assay for the determination of the efficiency of conjugation with Tetanic toxoid and hu-rec-EGF.

x axis: dilutions of conjugate.

y axis: optical density at 405 nm

Figure 11: antibody titer against hu-rec-EGF in non human primates immunized with hu-rec-EGF, coupled to tetanic toxoid.

Claims

1. Vaccine composition of autologous EGF, conjugated with any carrier protein, containing an adjuvant, able to produce an autoimmune reaction against the autologous Epidermal Growth Factor.

2. Vaccine composition according to claim 1, with the characteristic of containing human recombinant EGF

3. Vaccine composition according to claim 1, with the characteristic of containing as carrier protein the CTB (cholera toxin B chain).

4. Vaccine composition according to claim 1, with the characteristic of containing as carrier protein Tetanic Toxoid.

5. Vaccine composition according to claim 1, with the characteristic of containing as carrier protein a monoclonal antibody.

6. Vaccine composition according to claim 1, with the characteristic of containing as carrier protein Neisseiria meningitides outer membrane protein.

7. Vaccine composition according to claim 1, with the characteristic of containing as adjuvant Aluminium Hydroxide.

8. Use of the vaccine composition according to any one of claims 1 to 7 for the treatment of malignant diseases.

9. Use of autologous EGF of a mammal in the preparation of a vaccine for the treatment of EGF-related diseases.

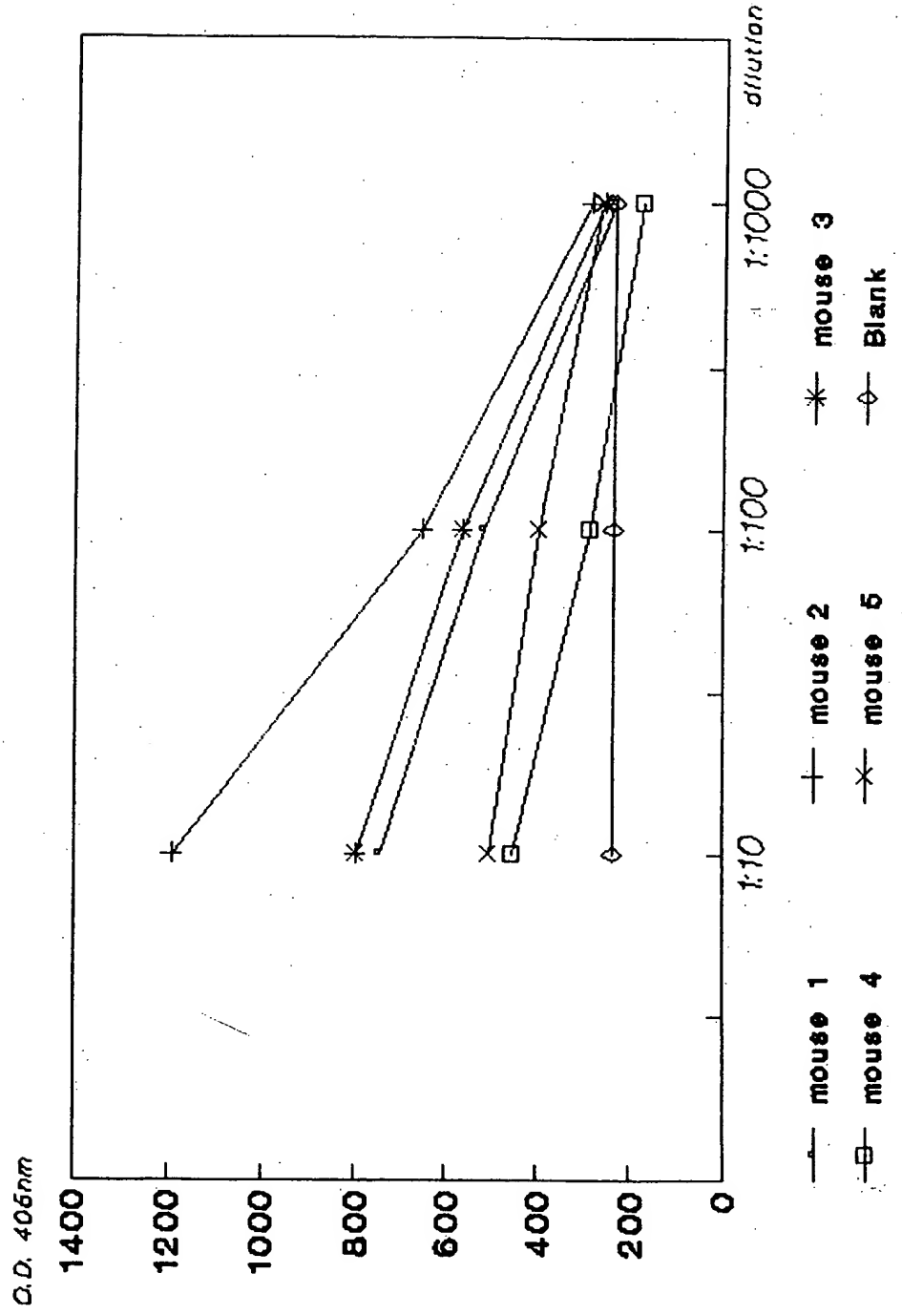
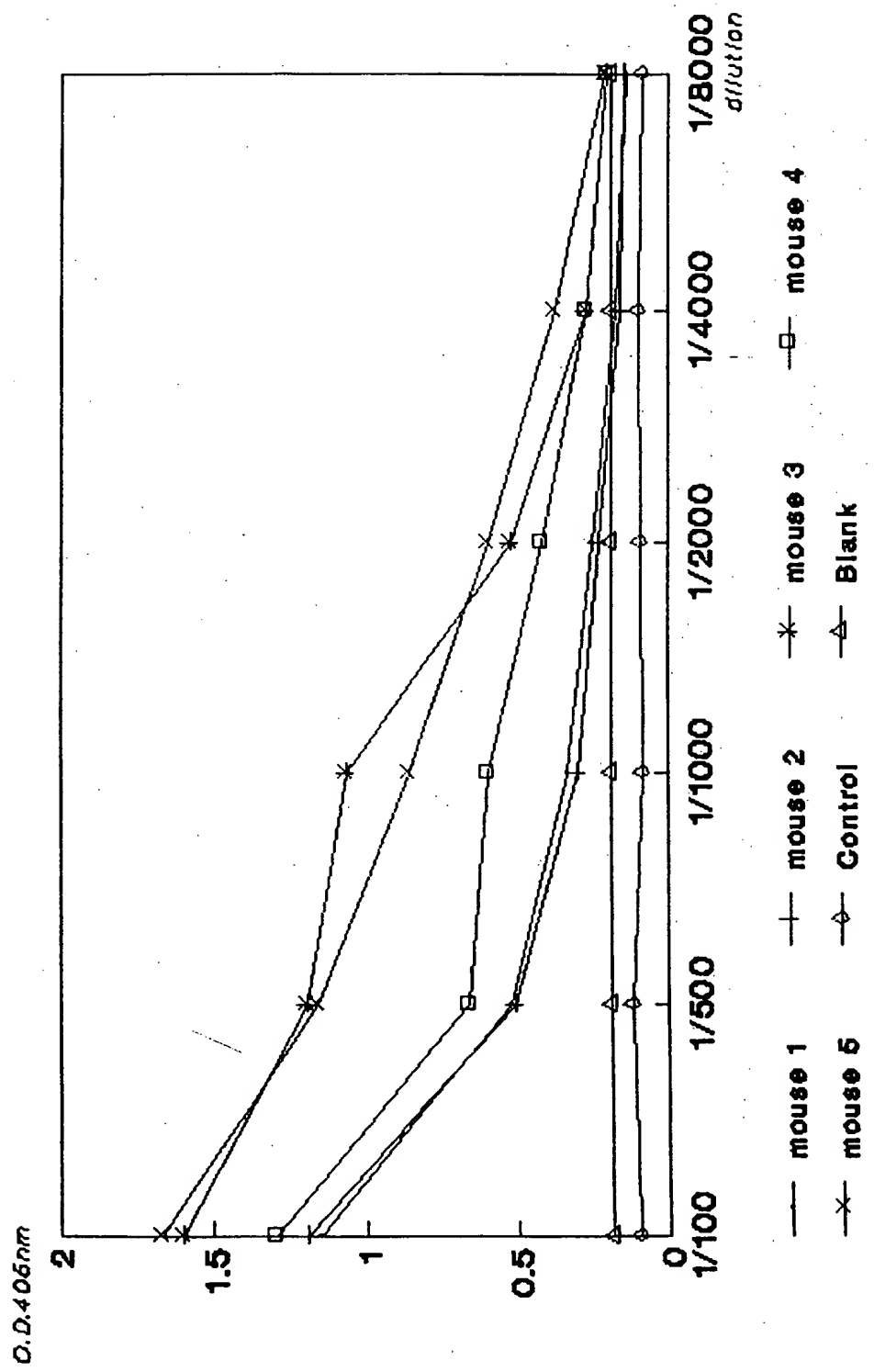

O.D. 405nm
1400
1200
1000
800
600
400
200
0
1:10
1:100
1:1000
dilution
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5
Blank

FIG.2


O.D.406nm
2
1.5
1
0.5
0
1/100
1/500
1/1000
1/2000
1/4000
1/8000
dilution
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mouse 2
mouse 3
mouse 4
mouse 5
Control
Blank

FIG. 4

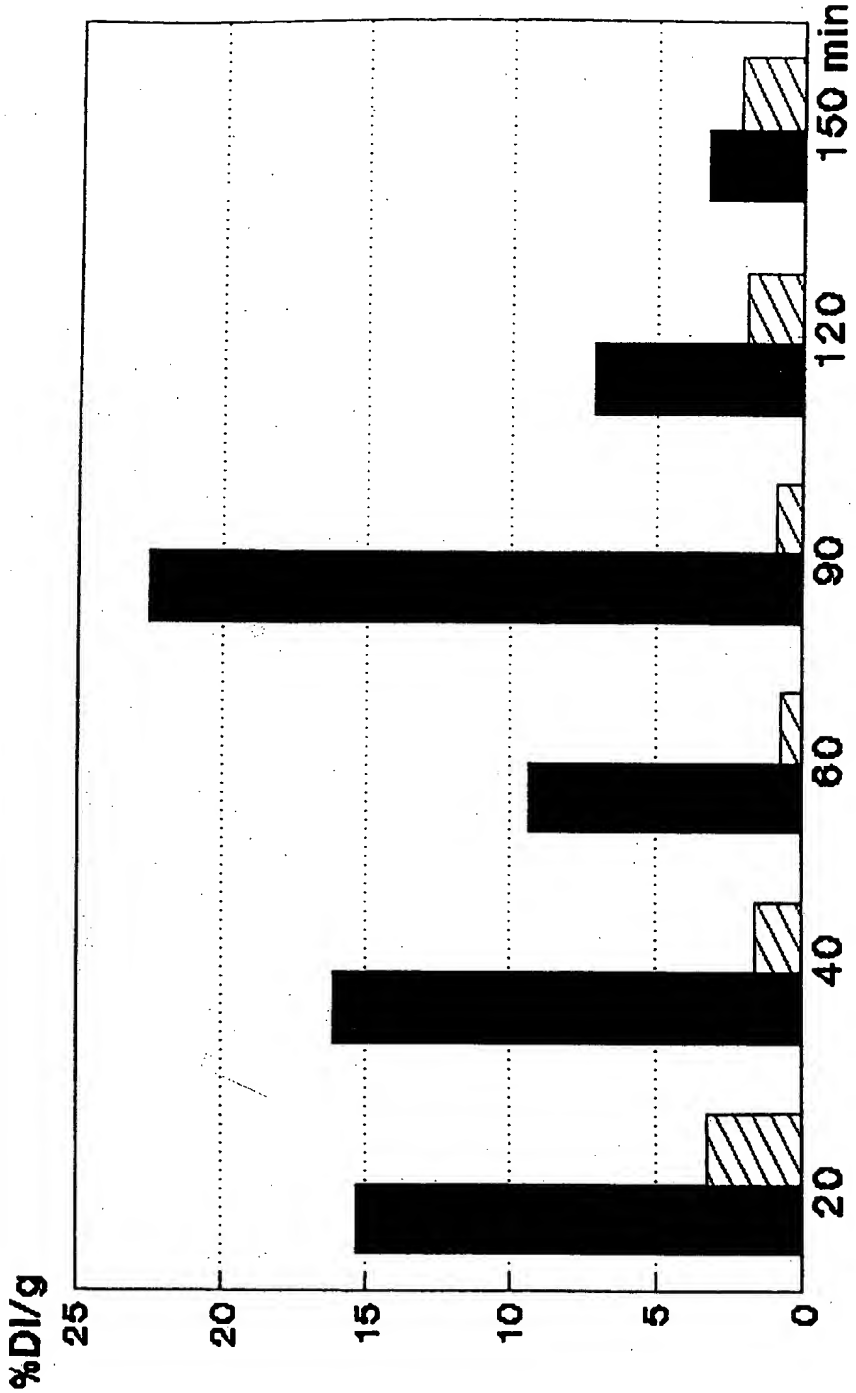

FIG.6a

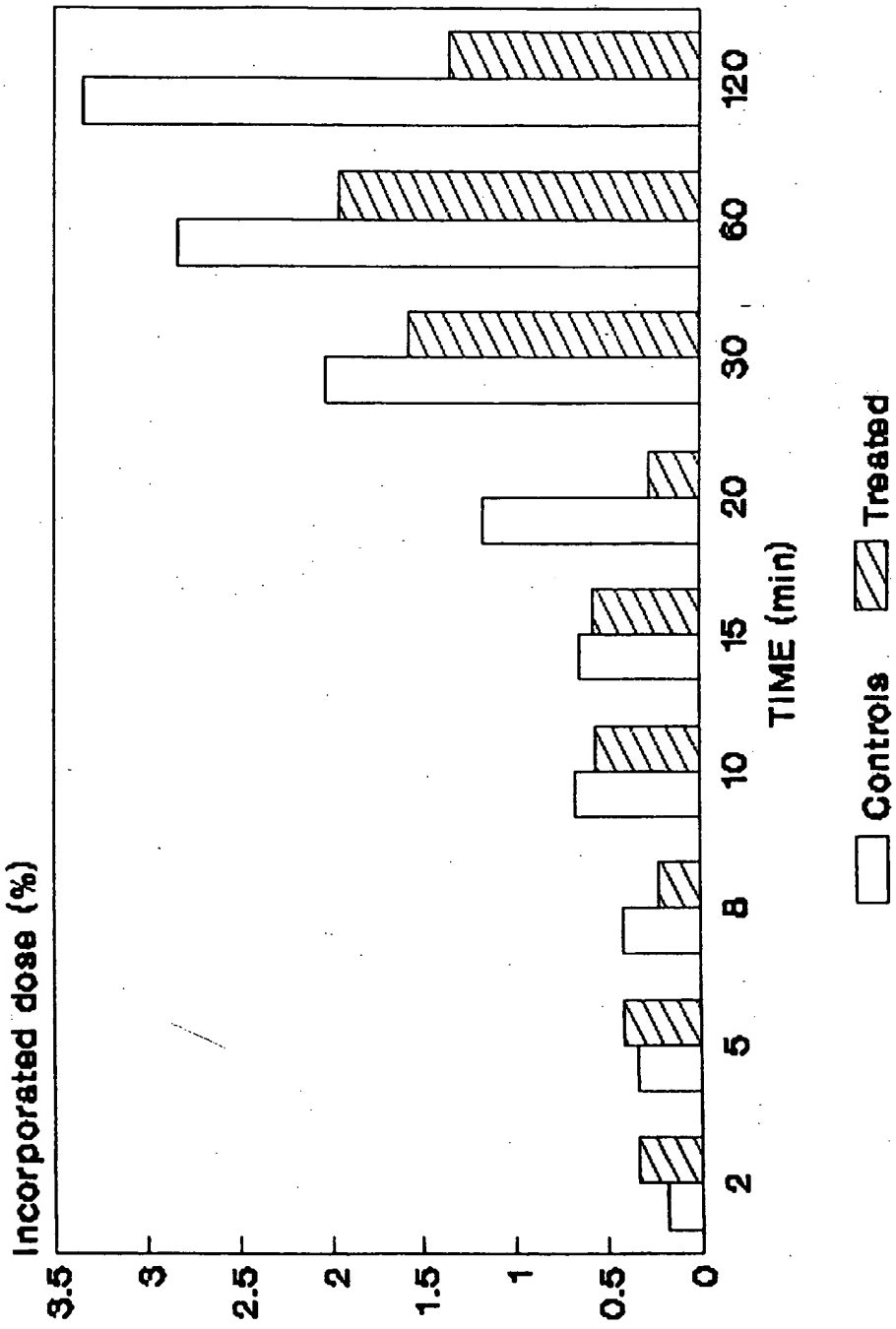
Incorporated dose (%)
3.5
3
2.5
2
1.5
1
0.5
0
2
5
8
10
15
20
30
60
120
TIME (min)
Controls
Treated

FIG.7

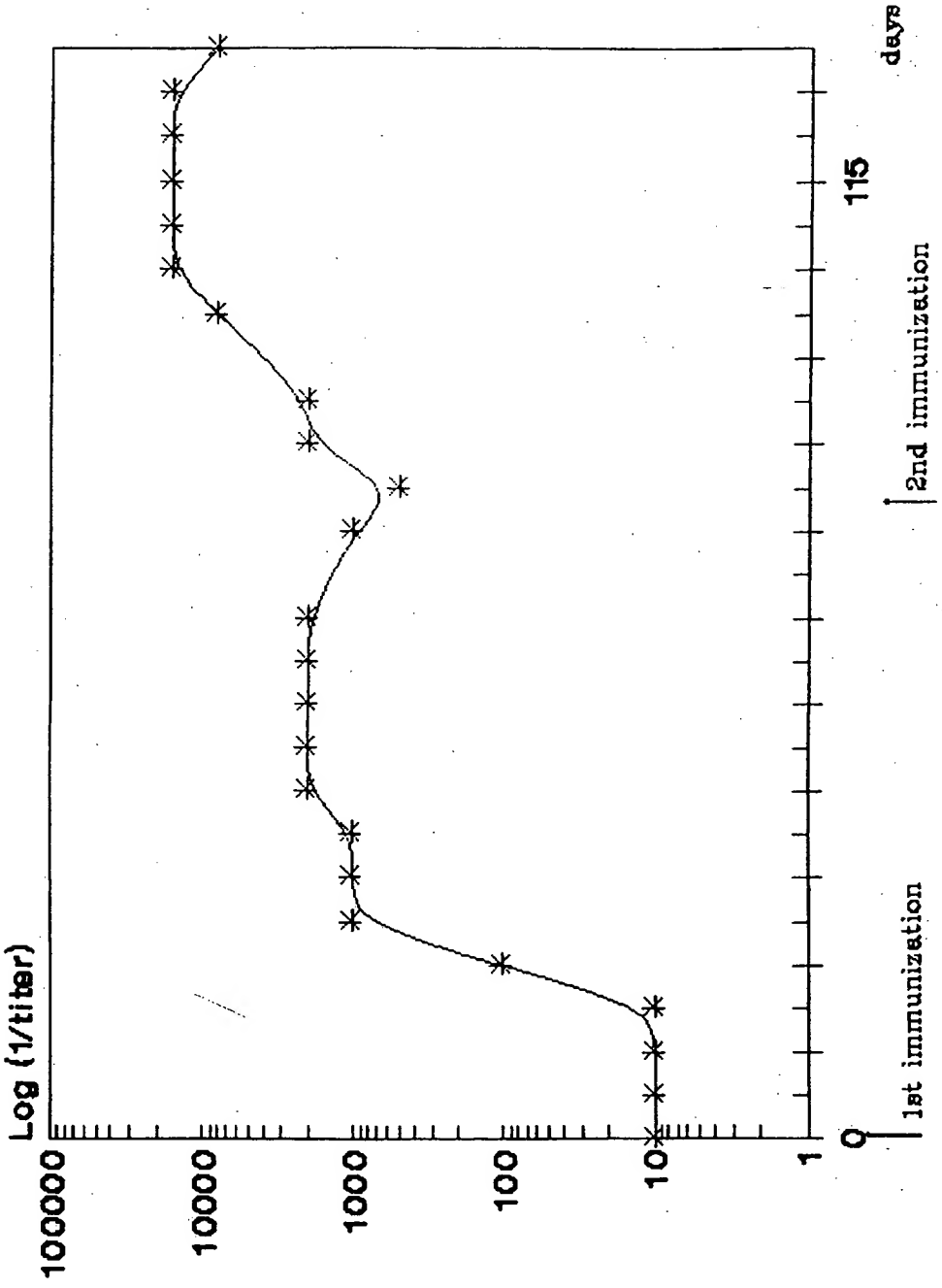
Log (1/titer)
100000
10000
1000
100
10
1
0
1st immunization
115
days
2nd immunization

FIG.9

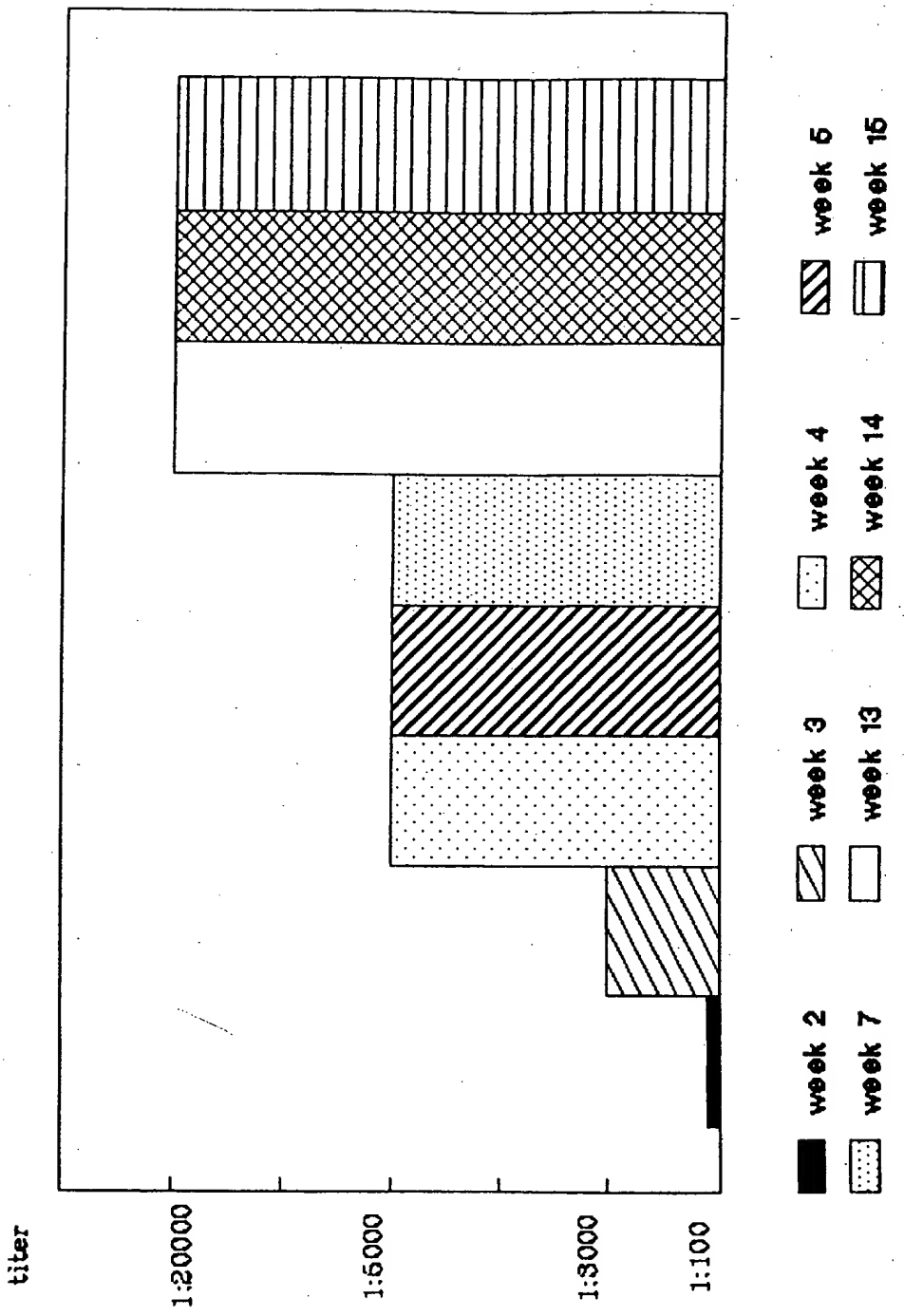
titer
1:20000
1:6000
1:3000
1:100
week 2
week 7
week 3
week 13
week 4
week 14
week 5
week 15

FIG.11

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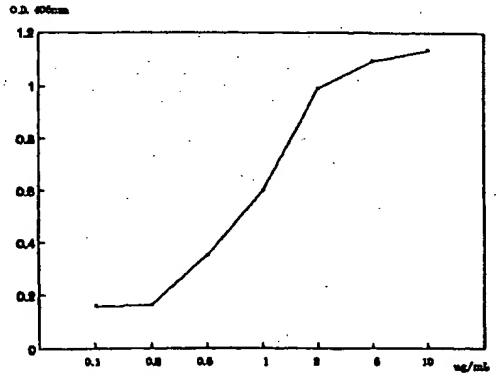

FIG.1

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INCOMPLETE SEARCH

"Remark: Although claim 8 is directed to a method of treatment of the human/animal body (Article 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition."